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Spatial Structure and Insertion Capacity of Immunodominant Region of Hepatitis B Core Antigen

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Summary
Spatial and immunochemical elucidation of hepatitis B core antigen suggestec unique organization of its major immunodominant region (MIR) localized within the central part of molecule around amino acid residues 74–83. This superficial loop was recognized as the most prospective target for the insertion of foreign epitopes ensuring maximal antigenicity and immunogenicity of the latter. MIR allowed a substantial capacity of insertions up to about 40 amino acid residues without loss of the capsid-forming ability of core particles. Vector capacity as well as structural behavior and immunological fate of inserted epitopes were dependent on their primary structure. Special sets of display vectors with retained but cross-sectioned MIR as well as with uni- and bidirectionally shortened MIR have been investigated.

Introduction

Hepatitis B core antigen (HBcAg) which was one of the first models of 'display vectors' for particle usage as vaccine carriers [1–5] attracted special interest from the viewpoint of its three-dimensional and immunological structure. According to early modelling data [6], the central part of the HBcAg molecule including amino acid residues 72–88 and predicted as a nonstructured loop has been used successfully for insertion of foreign peptide sequences ranging in length from 5 to 41 amino acid residues [5, 7–12]. Insertions have been done into the proposed tip of this loop where duck hepatitis B core antigen (DHBcAg) contained nonhomologous insertion of 39 amino acid residues [6], and namely between amino acid residues 77 and 78, 78 and 79 [Borisova et al., unpubl. data], 81 and 82 [7, 8, 12], 82 and 83 [13] as well as within gaps 75–81 [5, 11], 78–81 [10] and 78–82 [9]. It was hardly surprising that obtained chimeric core particles presented foreign epitopes on their surface and elicited strong B cell and Th cell responses against them, since the molecule region 74–83 was found in immunological investigations as presenting main HBcAg and hepatitis B e antigen (HBeAg) epitopes c1 and e1, respectively [14]. We designated this part of the molecule as a major immunodominant region (MIR) and concentrated our interest in its investigation on the combination of (1) the structural high resolution technique and (2) immunochemical methods of HBcAg epitope mapping. Although HBcAg still lacks an X-ray structure, recent electron cryomicroscopy [15] and epitope mapping [16] data are steps in the direction of the elaboration of the finest exposure strategy of foreign epitopes within HBcAg MIR.

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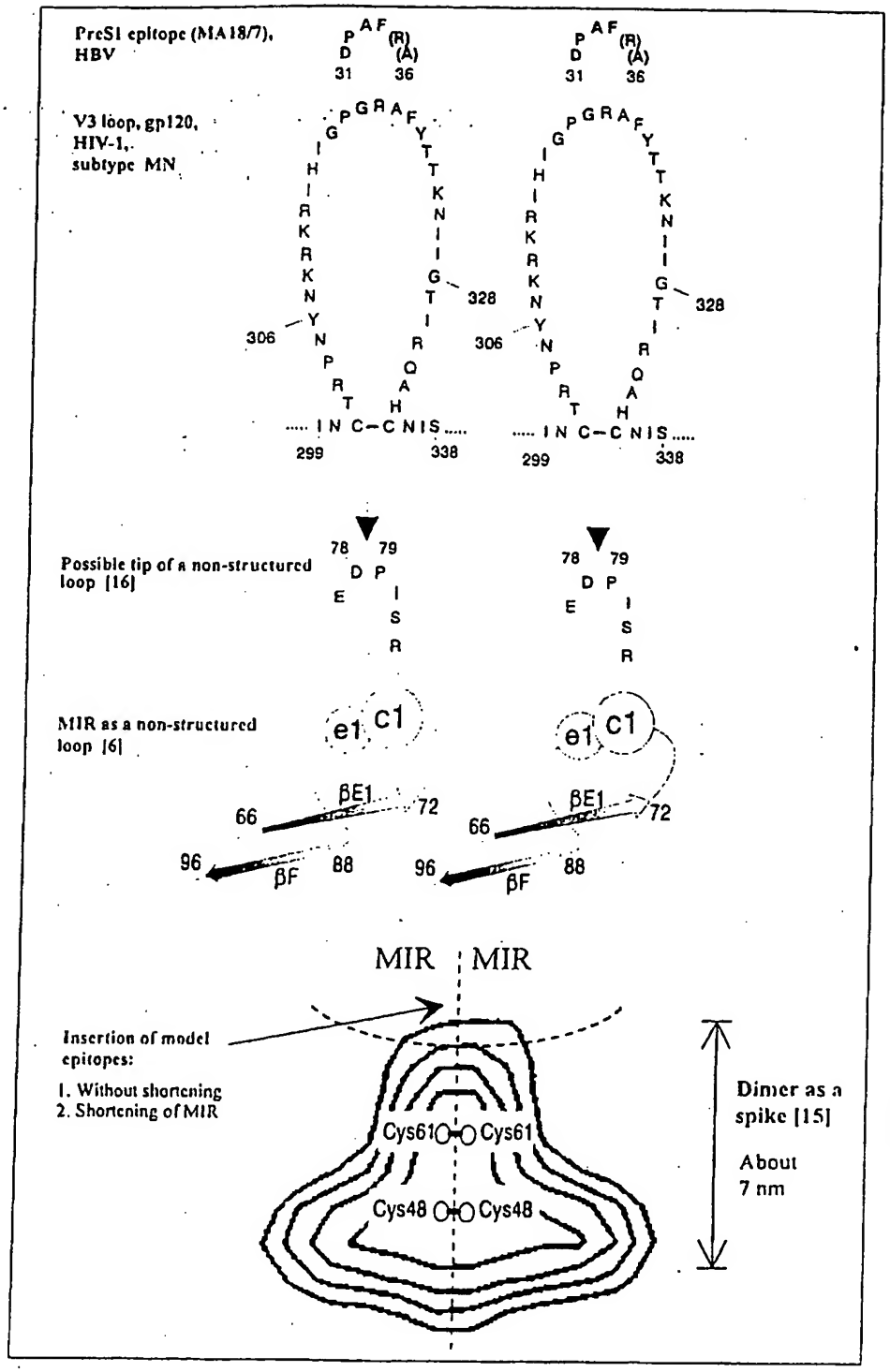

Fig. 1. General scheme of epitope display within MIR of HBcAg. The spike-like structure and dimensions of dimeric basal unit of HBcAg are taken from electron cryomicroscopy data [15]. Disulfide bonding between monomers is also indicated [23]. Homology-based prediction of MIR as a nonstructured loop 72–88 is shown according to Argos and Fuller [6]. Two model epitopes used for construction of universal display vectors are presented at the top. Numbers of V3 loop amino acid residues bounding the shortened epitope used by us (see table 1) are marked. PreS1 amino acid residues in brackets stand for additional, but not obligatory elements of the epitope.

Spatial Structure of HBcAg

First homology-based modelling of the HBcAg molecule adopted it to a T = 3 structural symmetry [6] confirming in principle early electron microscopy data [17] and served for a long time as an initial approximation of its structurally important regions (fig. 1). Recent electron-cryomicroscopic and image-processing investigations of the three-dimensional structure of core particles revealed two alternative pathways of molecule self-assembly into

T = 3 and T = 4 dimer-clustered packings, containing 180- and 240-protein subunits and being approximately 30 and 34 nm in diameter, respectively [15]. The three-dimensional map computed from images of small particles showed 90 protruding spikes and the corresponding map for the large particle showed 120 protruding spikes, both dimer-clustered. The arrangement of the holes means that the putative protein dimer has the shape of the head of a hammer with the protruding spike forming the 'handle' and the wedges of the shell domain extending towards the centers of the rings of 6 or 5 dimers forming the head [15].

Localization of Proper HBcAg Epitopes

In parallel with the determination of the spatial structure of the core particle [15], an attempt was made to map the surface of HBcAg particle with a set of monoclonal antibodies targeted to definite linear epitopes in an ELISA competition assay [16]. The competition technique was chosen, because other standard ELISA methods using support-bonded or antibody-captured antigens led in our hands to an overestimation of the surface accessibility of original and/or inserted epitopes, possibly on account of partial disassembly of the capsids on the solid phase. As a result, amino acid residues 78–83 within the proposed MIR were found truly surface-exposed in native particles. Almost all polyclonal, human as well as hyperimmune, and monoclonal anti-HBc antibodies which normally failed to recognize HBcAg peptides, or its disassembled and denatured forms, competed with monoclonal antibodies mapped against MIR [16, 18]. Therefore, clear evidence of (1) a unique superficial localization of MIR and (2) outstanding importance of its conformation regarding antigenicity and immunogenicity were obtained. It may be safely suggested that localization of MIR including so-called c1 and e1 epitopes [14] is connected in some way with protruding 7-nm-long spikes found by Crowther et al. [15] (fig. 1).

Insertion Strategy

We started our experiments of presenting viral epitopes on HBcAg with C-terminal insertions, using position 144 which was located close to the HBeAg border at positions 147 or 149 and seemed therefore spatially and functionally important [2, 4]. We also tried to examine positions 31, 96, 109 and 131–132 as potential targets [2, 19], but concentrated our efforts finally on MIR [9]. Since 39 C-terminal amino acid residues were found to be dispensable for self-assembly, we decided upon full-length and C-terminally truncated, so-called HBcΔ, carriers in favor of the latter (fig. 2). Among the advantages of HBcΔ the most important were (1) high level synthesis in *Escherichia coli* cells and (2) appearance as empty shells due to low affinity to nucleic acids, although HBcΔ were less stable than the corresponding full-length protein particles.

Specialized Display Vectors

Vectors are usually designed by the introduction of unique restriction sites into a chosen gene region or into two or more different regions allowing the simultaneous insertion of foreign epitopes into more than one target. We combined this approach with the immunological marking of possible insertion sites by the introduction of short polylinkers encoding 4- to 5-amino-acid-residue-long epitopes, sufficient for recognition by well-defined monoclonal antibodies (fig. 2). Such epitopes were referred to by us as 'immunological markers', and as the first of their kind epitope DPAF and its longer variants DPAFR and DPAFRA [20] recognized by murine monoclonal anti-preS1 antibody MA18/7 [21] were introduced. These markers were surrounded by unique restriction sites allowing (1) substitution of 'immunological marker' for the desired epitopes and (2) construction of further 'universal carriers' with shortened MIR.

In order to construct such 'universal carriers' having diminished or totally abolished HBc antigenicity and immunogenicity and enhanced vector capacity, we created a set of HBc genes with MIR, shortened by controlled uni- or bidirectional Bal31 digestion, but containing the appropriate 'immunological markers' (fig. 2). Such MIR-shortened HBc variants of different lengths which retained their capability for (1) high-level synthesis in *E. coli* and (2) self-assembly were selected for further use in protein-engineering and immunological studies. They may be used for creation of multivalent particles by simultaneous insertion of foreign sequences at MIR and amino acid position 144 where another polylinker remained.

Table 1 presents a summary of the MIR-inserted constructions that have been made so far in our laboratory. The capacity of vectors is relatively high, the largest inserted epitopes, domain 'a' of HBsAg and V3 loop of HIV-1 gp120, are about one third in length of the vector itself.

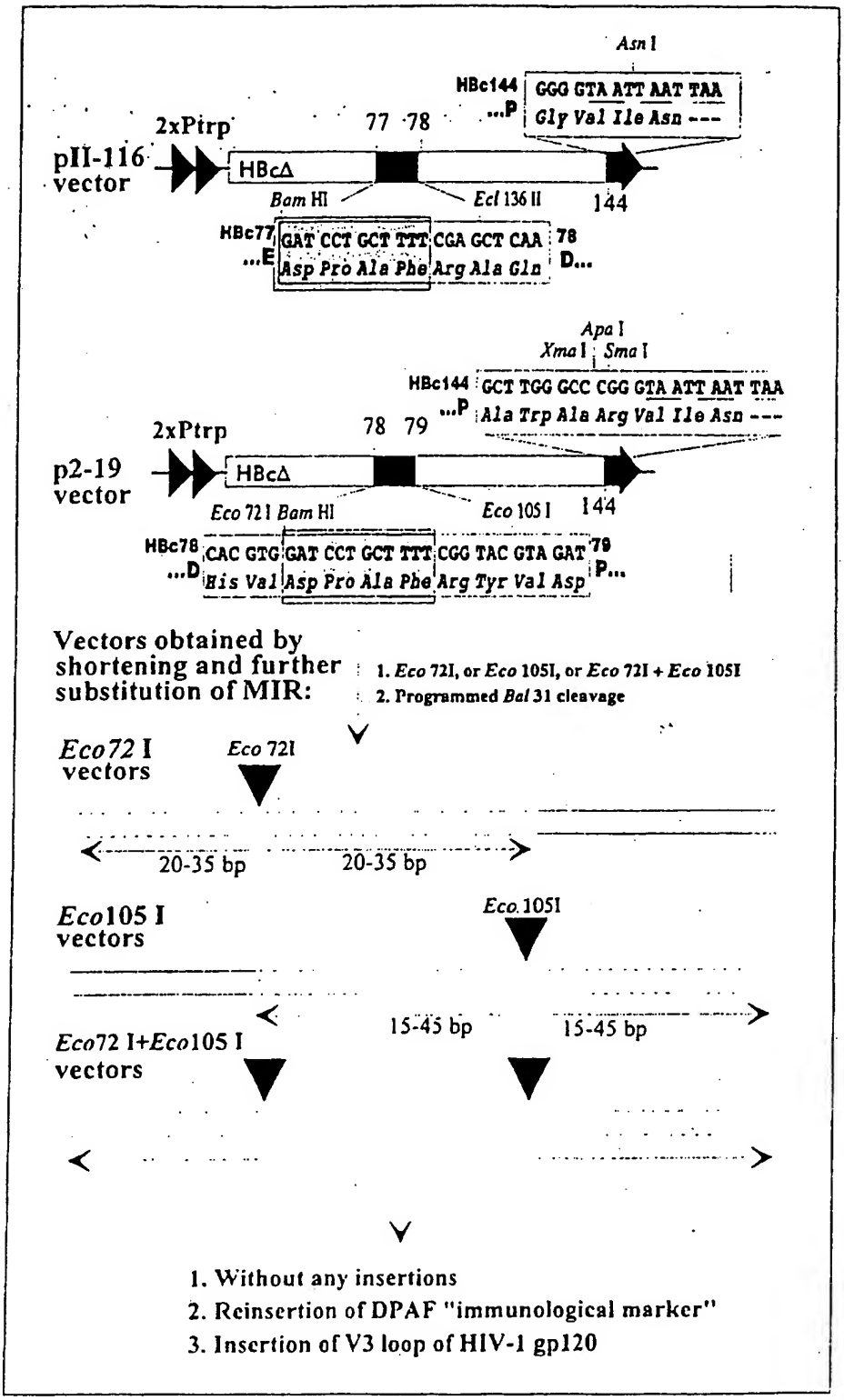

Fig. 2. Schematic structure of a family of HBcAg universal display vectors. Polylinkers inserted at positions 78 and 144 are depicted as nucleotide and corresponding peptide sequences. 'Immunological marker' represented by HBV preS1 MA18/7-recognized epitope is highlighted. Three groups of Bal31-shortened display vectors (1) without epitope insertions, (2) with preS1 epitope inserted, and (3) with V3 loop inserted are shown as summarized.

Table 1. Immunological properties of chimeric core particles with foreign insertions at MIR

Clone	Epitope source	structure/insertion site		length	Antigenicity[1] HBcAg	Antigenicity[1] epitope	B cell immunogenicity[2] anti-HBc	B cell immunogenicity[2] anti-epitope	T cell immunogenicity[3] HBcAg	T cell immunogenicity[3] epitope
HBV										
II-116	preS1(31–36)	...E77	DPAFRAq D78... P144 awarvin*	7 (7)	yes	yes	+++	+	+++	+
2-19	preS1(31–35)	...D78	hvDPAFRyvd P79...P144 awarvin*	10 (7)	yes	yes	NT	NT	NT	NT
GHS19	HBsAg(111–149)	...D78	qfPGSSTTSTGPCRTCTTPAQGTS MYPSCCCTKPSDGNCTC R82...	41 (7)	no	yes	++	++	+	+
		...P144	awarvin*							
O6-1	HBsAg(111–149)	...D78	qfPGSSTTSTGPCRTCTTPAQGTS MYPSCCCTKPSDGNCTC R82.....	41 (68)	no	yes	++	++	NT	NT
	preS2(1–54) (C-terminally)	...P144	krsiqaMQWNSTTFHQTLQDPRV RGLYFPAGGSSSGTVNPVPTTV SPISSIFSRIGDPALkrtshqds*							
HIV-1										
78-6	gp120(306–328), subtype MN	...D78	hYNKRKRIHIGPGRAFYTTKNI lGvd p79...	26 (7)	yes	yes	+++	+++	NT	NT
		...P144	awarvin*							

Peptide sequences delineate total number of inserted amino acid residues, HBcAg border amino acids are in bold print, non epitope random sequences on the gene junctions are shown as small letters, protein termination is marked with an asterisk. The length of peptide sequences added C-terminally is given in parentheses.

1 Antigenicity was determined using the double radial immunodiffusion test (HBcAg) and ELISA with epitope-specific antibodies (epitope).

2 Antibody titer in the range of 10^3 to 10^6 (+++), 5×10^2 to 10^3 (++) and 2×10^2 to 5×10^2 (+).

3 T cell proliferation index (10 days after immunization) in the range of 15–25 (+++), and 2.5–5 (+).

Antigenicity

Antigenic features of proper and inserted sequences are inevitably dependent upon their superficial accessibility and correct conformation. In contrast to C-terminal insertions, internal MIR-inserted epitopes were unambiguously characterized as located on the surface of chimeric particles. This statement was verified not only by direct antigen-bonded or antibody-captured ELISA and immunogold electron microscopy, but also by competitive ELISA which made it possible to discriminate between 'fully' and 'partially' exposed epitopes. Internal insertions into MIR seemed to be optimal immunologically because they provided (1) the highest possible epitope density on the surface of chimeric core particle and (2) the necessary epitope mobility to achieve its correct conformation. The internal loop was used therefore not only for the exposure of linear epitopes, but also for mimicking of typically conformational epitopes such as, for example, determinant 'a' of HBsAg (table 1). Although HBs antigenicity of chimeras was significantly lower than that of native particulate HBsAg, this finding pioneered the possibility of artificial modelling of conformational epitopes. It is worth noticing that determinant 'a' sequence added to position 144 of the HBcAg molecule was unable to mimick HBsAg [22].

Dissection of the major c1 epitope resulted in declined HBc antigenicity (and immunogenicity). In this regard the deletion variants of MIR are of particular interest. Attempts were made to insert simultaneously two different epitopes into different HBc protein target regions. Chimeras exposing HBsAg determinant 'a' internally and preS2 C-terminally were found antigenic for both inserts (table 1).

B Cell Immunogenicity

Like antigenic properties, B cell immunogenicity of inserted epitopes seemed to be strongly dependent upon their superficial location. It stood to reason that insertion of foreign epitopes at MIR allowed a higher antiepitope response in comparison with other insertion targets studied [5, 7–13]. In spite of dissection of MIR, chimeric particles constructed without deletion of proper amino acid residues in MIR induced nevertheless strong anti-HBc

response (table 1). As in the case of C-terminal insertions, HBcAg-specific antibodies induced by chimeric particles competed with polyclonal human and hyperimmune anti-HBc antibodies and were targeted therefore to the conformational c1 epitope (table 1). The situation changed when some MIR amino acid residues were deleted. In the case of the insertion of the HBsAg 'a' epitope accompanied by a deletion of amino acids 79-PIS-81, induced anti-HBc antibodies did not compete with polyclonal anti-HBc antibodies and were targeted therefore to HBcAg regions other than the c1 epitope [9]. Preliminary investigations of MIR-shortened variants (fig. 2) strengthened our idea of chimeric core particles devoid of major HBc immunogenicity. As in the case of C-terminal insertions, internally inserted epitopes ensured strong antibody response only in the capsid, but not in the denatured form.

T Cell Immunogenicity

Since the T cell immunogenicity of inserted epitopes needs not be exclusively dependent upon their surface accessibility, it was of direct interest to compare the appropriate capacities of internal and C-terminal insertions. As illustrated in table 1, chimeric core particles ensured strong HBc-specific T cell help, when all MIR amino acid residues were conserved (clone II-116), but behaved as weak T immunogen in the case of 79-PIS-81 deletion (clone GHS19). It is noteworthy that short internal insertion of 6 amino acid residues (clone II-116) ensured a remarkable T cell response, like the longer HBsAg 'a' epitope (clone GHS19) which induced a T cell response not only to the appropriate HBsAg peptide but also to native HBsAg. Therefore, internal insertions provided some epitope-specific T cell help but did not show any advantages in comparison with C-terminal insertions in this respect [9].

Conclusions

There are good reasons to believe that amino acid residues 74–83 involved in the HBcAg major immunodominant region are localized superficially on 7-nm-long protruding spikes of core particles revealed recently by electron cryomicroscopy. These amino acids play an important role in the architecture of the conformational c1 epitope of HBcAg and determine most immunological properties of the latter. It is not surprising that this sequence predicted as a nonstructured loop shows considerable promise for the insertion of foreign epitopes. In fact, internally inserted epitopes show maximal antigenicity as well as B and T cell immunogenicity in comparison with other insertion targets. Existing display vectors exhibit insertion capacity sufficient for mimicking of the architecture of not only linear but also conformational epitopes. Construction of special sets of display vectors with shortened MIR allowed us to start with core particles having diminished or cleared proper HBcAg antigenicity and immunogenicity.

References

1 Clarke BE, Newton SE, Carroll AR, Francis MJ, Appleyard G, Syred AD, Highfield PE, Rowlands DJ, Brown F: Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. Nature 1987;330:381–384.

2 Borisova G, Bundule M, Grinstein E, Dreilina D, Dreimane A, Kalis J, Kozlovskaya T, Loseva V, Ose V, Pumpen P, Pushko P, Snikere D, Stankevica E, Tsibinogin V, Gren EJ: Recombinant capsid structures for exposure of protein antigenic epitopes. Mol Gen Life Sci Adv 1987; 6:169–174.

3 Stahl SJ, Murray K: Immunogenicity of peptide fusions to hepatitis B virus core antigen. Proc Natl Acad Sci USA 1989;86:6283–6287.

4 Borisova GP, Berzins I, Pushko PM, Pumpen P, Gren EJ, Tsibinogin VV, Loseva V, Ose V, Ulrich R, Siakkou H, Rosenthal HA: Recombinant core particles of hepatitis B virus exposing foreign antigenic determinants on their surface. FEBS Lett 1989;259:121–124.

5 Schodel F, Moriarty AM, Peterson DL, Zheng J, Hughes JL, Will H, Leturcq DJ, McGee JS, Milich DR: The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. J Virol 1992;66:106–114.

6 Argos P, Fuller SD: A model for the hepatitis B virus core protein: Prediction of antigenic sites and relationship to RNA virus capsid proteins. EMBO J 1988;7:819–824.

7 Brown AL, Francis MJ, Hastings GZ, Parry NR, Barnett PV, Rowlands DJ, Clarke BE: Foreign epitopes in immunodominant regions of hepatitis B core particles are highly immunogenic and conformationally restricted. Vaccine 1991;9:595–601.

8 Yon J, Rud E, Corcoran T, Kent K, Rowlands D, Clarke B: Stimulation of specific immune responses to simian immunodeficiency virus using chimeric hepatitis B core antigen particles. J Gen Virol 1992;73:2569–2575.

9 Borisova G, Arya B, Dislers A, Borschukova O, Tsibinogin V, Skrastina D, Eldarov M, Pumpens P, Skryabin KG, Grens E: Hybrid hepatitis B virus nucleocapsid bearing immunodominant region from hepatitis B virus surface. J Virol 1993;67:3696–3701.

10 Von Brunn A, Brand M, Reichhuber C, Morys-Wortmann C, Deinhardt F, Schodel F: Principal neutralizing domain of HIV-1 is highly immunogenic when expressed on the surface of hepatitis B core particles. Vaccine 1993;11: 817–824.

11 Schodel F, Wirtz R, Peterson D, Hughes J, Warren R, Sadoff J, Milich D: Immunity to malaria elicited by hybrid hepatitis B virus core particles carrying circumsporozoite protein epitopes. J Exp Med 1994;180:1037–1046.

12 Tindle RW, Herd K, Londono P, Fernando GJP, Chatfield SN, Malcolm K, Dougan G: Chimeric hepatitis B core antigen particles containing B- and Th-epitopes of human papillomavirus type 16 E7 protein induce specific antibody and T-helper responses in immunised mice. Virology 1994;200:547–557.
13 Schodel F, Weimer T, Will H, Milich D: Recombinant HBV core particles carrying immunodominant B-cell epitopes of the HBV preS2-region; in Brown F, Chanock RM, Ginsberg HS, Lerner RA (eds): Vaccines 90. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1990, pp 193–198.
14 Salfeld J, Pfaff E, Noah M, Schaller H: Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. J Virol 1989;63:798–808.
15 Crowther RA, Kiselev NA, Bottcher B, Berriman JA, Borisova GP, Ose V, Pumpens P: Three dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. Cell 1994;77:943–950.
16 Pushko P, Sallberg M, Borisova G, Ruden U, Bichko V, Wahren B, Pumpens P, Magnius L: Identification of hepatitis B virus core protein regions exposed or internalized at the surface of HBcAg particles, by scanning with monoclonal antibodies. Virology 1994;202:912–920.
17 Onodera S, Ohori H, Yamaki M, Ishida N: Electron microscopy of human hepatitis B virus cores by negative staining-carbon film technique. J Med Virol 1982;10:147–155.
18 Bichko V, Schodel F, Nassal M, Gren E, Berzins I, Borisova G, Miska S, Peterson DL, Gren E, Pushko P, Will H: Epitopes recognized by antibodies to denatured core protein of hepatitis B virus. Mol Immunol 1993;30:221–231.
19 Berzins I, Ulrich R, Meisel H, Kruger DH, Borisova G, Pumpens P, Grens E, Gelderblom H, Ladhoff AM, Schnitzler P, Darai G, Bautz EKF: Sites in the core antigen of HBV allowing insertion of foreign epitopes; in Vaccines. Cold Spring Harbor, Cold Spring Harbor Laboratory, 1994, pp 301–308.
20 Sominskaya I, Pushko P, Dreilina D, Kozlovskaya T, Pumpen P: Determination of the minimal length of preS1 epitope recognized by a monoclonal antibody which inhibits attachment of hepatitis B virus to hepatocytes. Med Microbiol Immunol 1992;181:215–226.
21 Heermann KH, Goldmann U, Schwartz U, Seyffarth T, Baumgarten H, Gerlich WH: Large surface proteins of hepatitis B virus containing the pre-S sequence. J Virol 1984;52:396–402.
22 Stahl SJ, Murray K: Immunogenicity of peptide fusions to hepatitis B virus core antigen. Proc Natl Acad Sci USA 1989;86:6283–8287.
23 Zheng J, Schodel F, Peterson DL: The structure of hepadnaviral core antigens. Identification of free thiols and determination of the disulfide bonding pattern. J Biol Chem 1992;267:9422–9429.

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